Supporting Information

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SI Text

Lung Tumor Tissue Disaggregation. Solid tissues were finely minced by razorblade, washed in DMEM/F12 (Lonza), and then incubated with Accumax 1× (Innovative Cell Technologies) for 1 h at 37 °C. Single-cell suspension was obtained by filtering digested tissue through a 70-μm cell strainer and then gently loaded onto a layer of Histopaque-1077 gradient (Sigma–Aldrich). After centrifugation at 400 × *g* for 30 min at room temperature, red blood cells, dead cells, and debris were removed from the bottom of the tube, and live nucleated cells were collected at the interface.

Magnetic and Cytofluorimetric Cell Separation. ***CD133 separation.*** $CD133^+$ and $CD133^-$ cells were isolated from tumor cell suspension or sphere cultures by FACS or magnetic bead sorting using the MACS system (Miltenyi Biotech). Magnetic bead separation was used for the cases with a high content of $CD133^+$ cells (>10%).

For magnetic separation, cells were incubated with the monoclonal CD133/1 antibody labeled with MicroBeads (Miltenyi Biotech) for 30 min at 4 °C, and $CD133^+$ cells were selected by using MS columns (Miltenyi Biotech), which retained positive cells linked by beads. For FACS separation, cells were stained with CD133/1-PE antibody (AC133 clone; Miltenyi Biotech) diluted 1:10 in blocking buffer solution for 10 min at 4 °C and were sorted with FACS Vantage-SE cell sorter (Becton Dickinson). In both instances, the purity of the $CD133^+$ and $CD133^-$ cell populations was evaluated by standard flow cytometry analysis using a PE-labeled antibody against human CD133/2 (clone 293C3; Miltenyi Biotech).

Mouse cell depletion. Xenograft tumor cell mixture was incubated with anti-mouse biotinylated $H\text{-}2K^d$ antibody (clone SF1-1.1; BD Bioscience) at 1 μg per 10^6 cells for 10 min at 4 °C and then incubated with 25 μg per 10^6 cells of anti-biotin Dynabeads for 20 min at 4 °C. Bound human cells were collected by using a magnet (Dynal MPB; Invitrogen).

IHC. Paraffin sections (2 μm thick) were dewaxed in xylene and rehydrated with distilled water. CD133 antigen retrieval was performed in citrate buffer solution (5 mM/L, pH 6). After peroxidase inhibition with a solution of 0.3% H_2O_2 in methyl alcohol for 30 min, the slides were incubated with the following antibodies: CD133/1 (AC133; Miltenyi Biotech); PE-10, TTF-1, and MIB-1 (Dako); CK-7 (NeoMarkers); and a pool for low-molecular weight cytokeratins (35βh11 from Dako and CAM5.2 from Becton Dickinson) and high-molecular weight cytokeratins (34βe12 from Dako and KS8.12 from Sigma). The primary antibody detection was performed by using Ultra Vision detection system-HPR polymer (Thermo Fisher Scientific) and diaminobenzidine substrate chromogen (Dako), followed by counterstaining with hematoxylin.

Real-Time PCR. Total RNA extraction from cells and DNase digestion were carried out with the RNAeasy kit (Qiagen). cDNA synthesis was performed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using 1 μg of total RNA in final volume of 20 μL.

The relative quantification of the stemness-associated genes mRNA (Notch1, Notch2, Hes1, SHH, Gli-1, CD133, Oct4/3, CXCR4, ITGA6, Nanog) was performed by TaqMan technology using the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) and ready-to-use Assay-on-Demand (Applied Biosystems).

Human HPRT was used as endogenous control for the normalization of different samples and relative quantization of gene expression; the data were analyzed by comparative C_t method ($\Delta\Delta C_t$). For analysis of xenograft mRNA, we previously validated the specificity for human transcripts of all used TaqMan assays (Applied Biosystems) to exclude a possible cross-reactivity with mouse transcripts.

Analysis of ABC transporter mRNA levels was performed with TaqMan Low-Density Arrays: 2 μL of cDNA was mixed with 48 μL of nuclease-free water and 50 μL of TaqMan Universal PCR Master Mix and then loaded into a sample port of the Micro Fluidic Cards (Human ABC Transporters Panel; Applied Biosystems). The thermal cycling conditions of real-time PCR were 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 30 s at 97 °C and 1 min at 59.7 °C. Data of RQ assays were analyzed with RQ Manager 1.2 and Sequence Detection System (SDS) 2.2.2 software (Applied Biosystems).

PKH26 and PKH67 Labeling. A549 and LT73 lung cancer spheres were labeled with the PKH26 red fluorescent and PKH67 green fluorescent dyes (Sigma). Briefly, harvested cells were dissociated to single cells, washed in PBS, and resuspended in 1 mL of the dilution buffer. The cell suspension was mixed with an equal volume of the labeling solution containing 4×10^{-6} M PKH26 or PKH67 in the dilution buffer and was incubated for 5 min at room temperature. A total of 2 mL of FBS was added to labeling solution to stop the reaction, and cells were washed three times in serum-free medium. The labeling ratio was determined by flow cytometry.

Red- and green-labeled single cells were mixed and plated at clonal density of 1×10^4 cells per mL in six-well culture plates (Corning). A distinct red or green fluorescent staining of growing spheres was monitored through fluorescence microscopy, indicating a clonal origin from one PKH26- or PKH67-labeled cell.

Cell Lines and Cell Sensitivity to Cisplatin. A549 and A549/Pt cells were maintained in RPMI medium 1640 plus 10% FCS. The cisplatin-resistant A549/Pt subline was generated from parental cells by continuous exposure to increasing concentrations of cisplatin. Resistance was stable up to 6 months when cells were grown in the absence of drug. Cellular sensitivity to drug was evaluated by using growth inhibition assays. For assessment of modulation of $CD133^+$ levels by cisplatin, exponentially growing A549 cells were seeded in T75 flasks, and 24 h later they were exposed for 1 h to a cisplatin concentration corresponding to IC_{80}. Cells were harvested 72 h after treatment for flow cytometric analysis.

In Vivo Evaluation of Response to Cisplatin. Tumor fragments were implanted at day 0, and tumor growth was followed by biweekly measurement of tumor diameters with a Vernier caliper. Tumor volume (TV) was calculated according to the formula: TV (mm^3) = $d^2 \times D/2$, where d and D are the shortest and the longest diameters, respectively. Cisplatin (Teva Pharma) was administered i.v. (10 mL/kg) at its optimal dose according to a schedule of every seventh day for three times (5 mg/kg per injection), starting when tumors were palpable (≥50 mm^3). The efficacy of drug treatment was assessed as TV inhibition percentage (TVI%) in treated over control mice, calculated as: 100 − (mean TV-treated mice/mean TV control mice × 100). For tumor removal, mice were killed by cervical dislocation under light anesthesia.

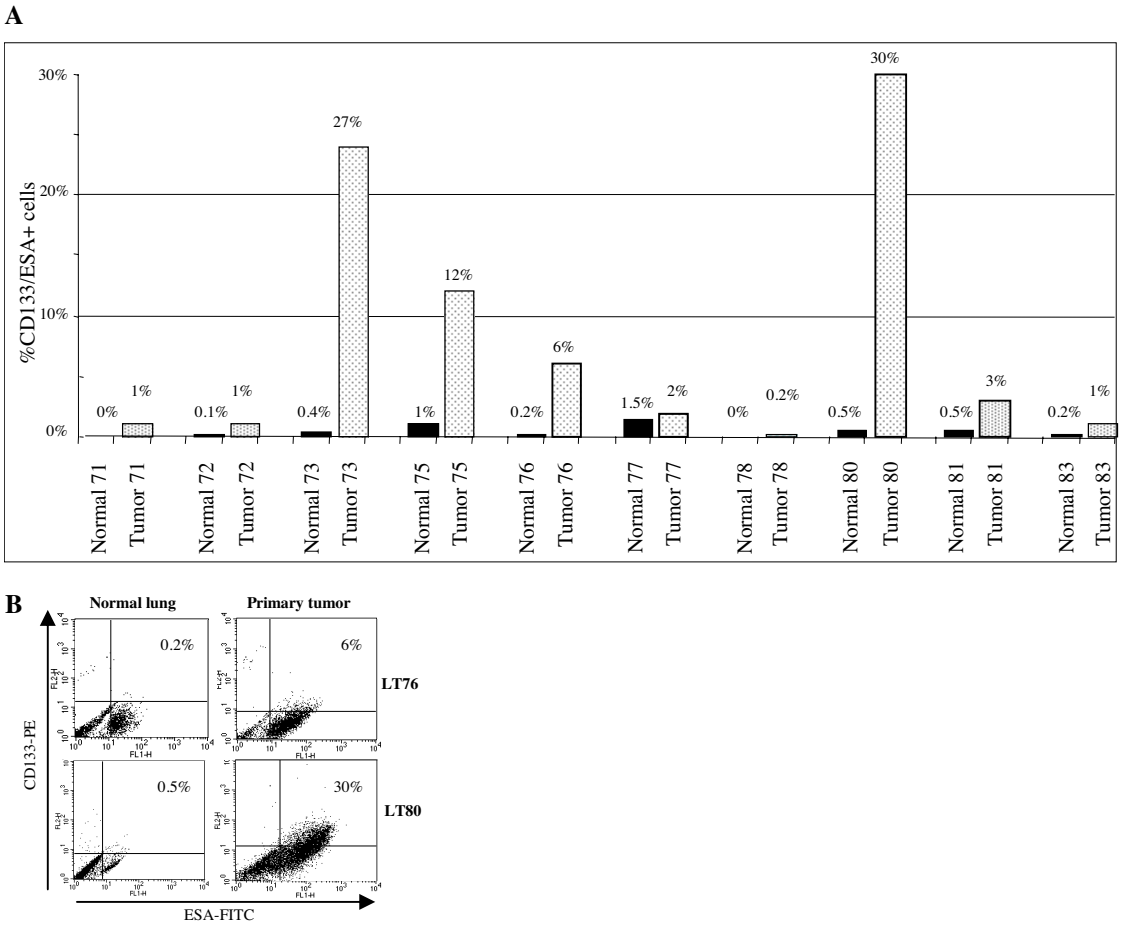

Fig. S1. Identification of CD133$^+$ cells in lung tumors and normal lung tissues. (*A*) Percentage of CD133$^+$ESA$^+$ cells in 10 primary tumors and corresponding normal lung tissues. (*B*) FACS analysis of CD133$^+$ESA$^+$ cells in two representative freshly dissociated lung tumors and corresponding normal tissue.

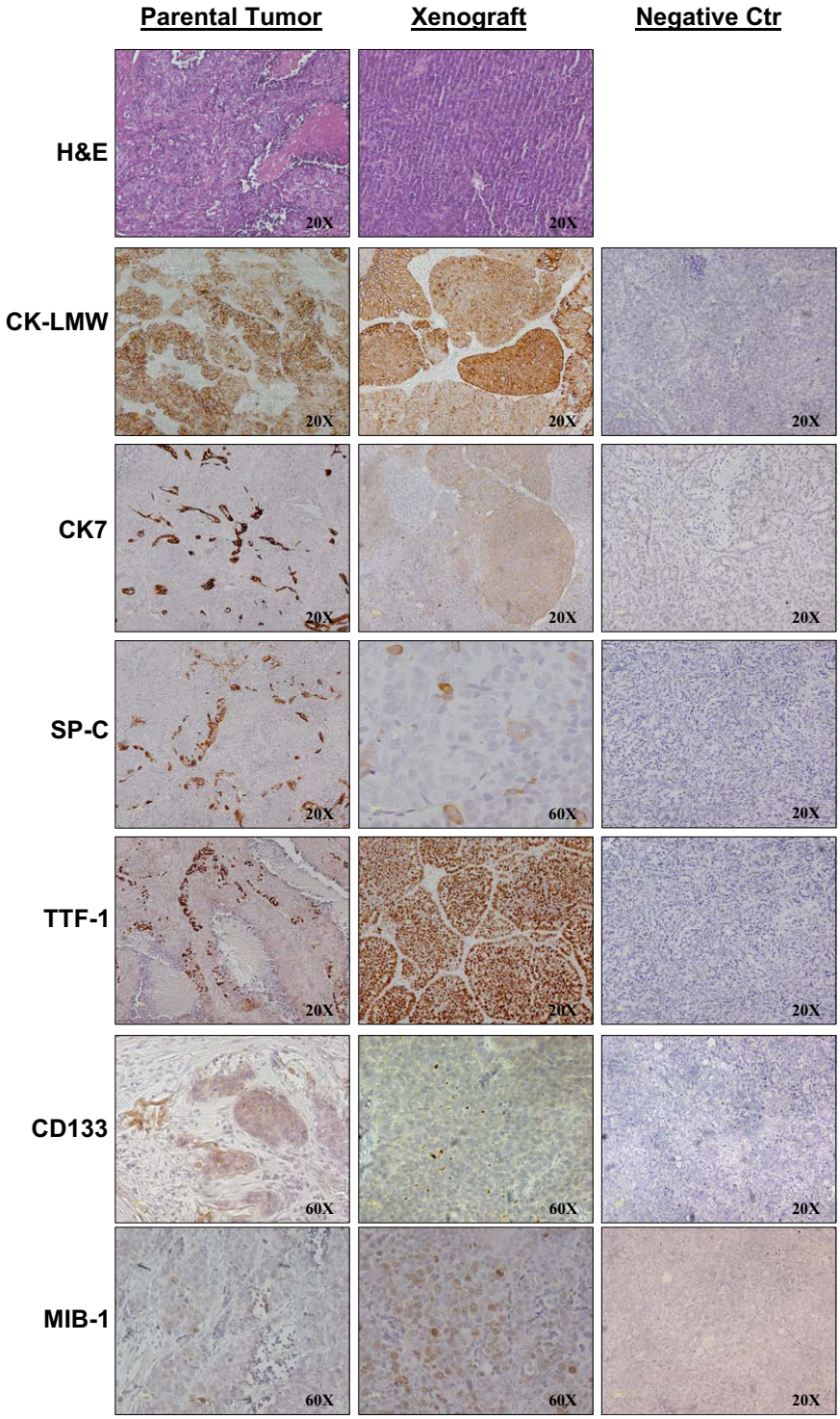

Fig. S2. IHC for low-molecular weight CKs (CK-LMW), cytokeratin 7 (CK7), surfactant protein C (SP-C), transcription thyroid factor 1 (TTF-1), CD133, and MIB-1 performed in a large cell carcinoma parental tumor (LT56) and corresponding xenograft. Control antibody-stained tumor sample is also shown (Negative Ctr).

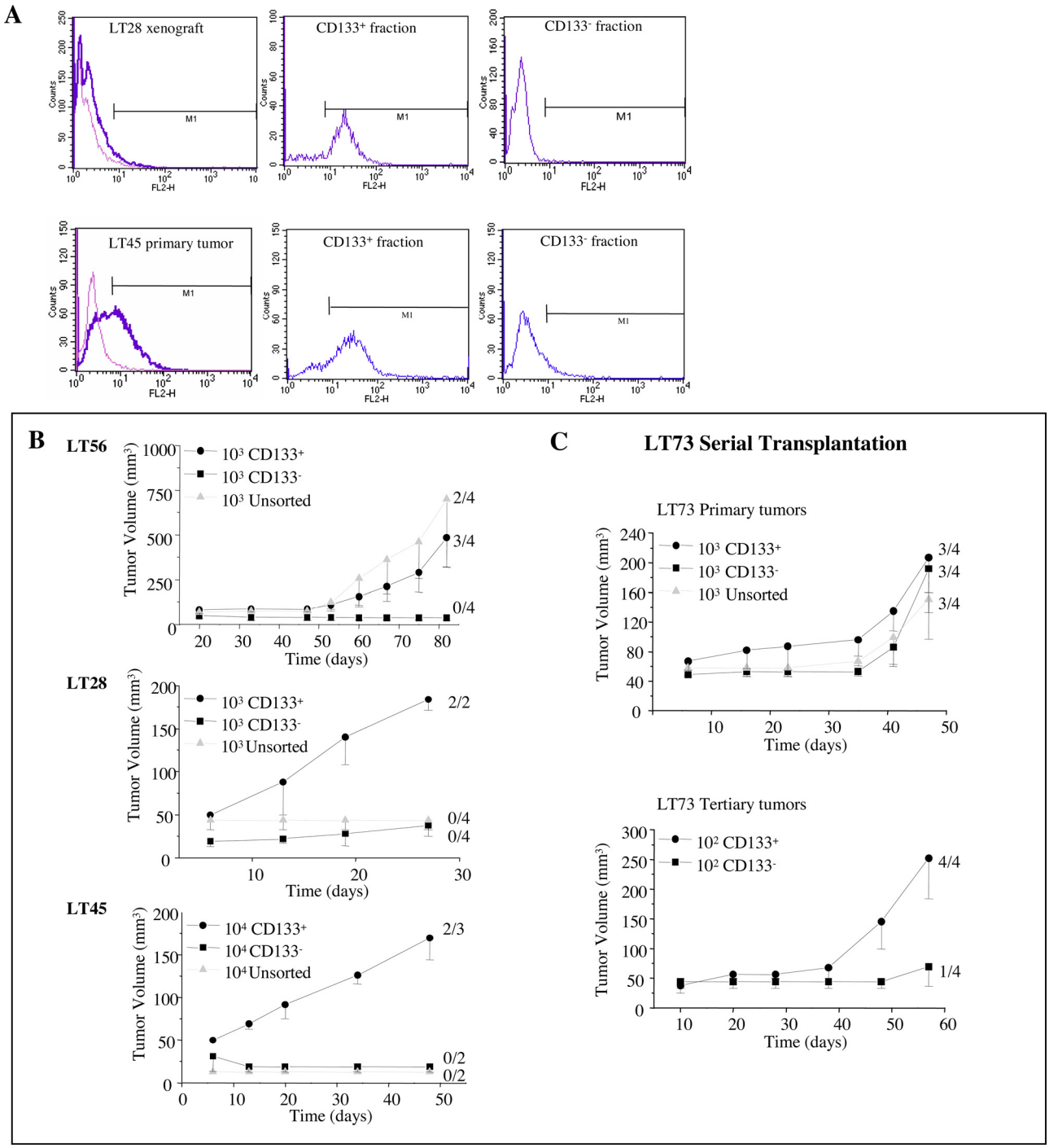

Fig. S3. (*A*) FACS analysis of CD133 expression before and after sorting of $CD133^+$ and $CD133^-$ fractions. $CD133^+$ cells were isolated from freshly dissociated lung tumors by FACS (LT28; *Upper*) or by magnetic beads (LT45; *Lower*). (*B*) In vivo tumorigenicity of $CD133^+$ and $CD133^-$ cells. Tumorigenic potential of $CD133^+$, $CD133^-$, and unsorted cells, purified from two xenografts (LT56 and LT28) and one primary tumor (LT45), after s.c. injection in immunocompromised mice. The tumor take for each subpopulation of injected cells is indicated. (*C*) In vivo serial transplantation assay. A total of 10^3 $CD133^+$ and $CD133^-$ cells, purified from LT73 xenograft, were injected s.c. into SCID mice. Derived tumor xenografts were dissociated to single-cell suspension and then serially reinjected in mice (10^2 cells), generating secondary and then tertiary tumors. Tumor growth curves of primary and tertiary tumors are shown.

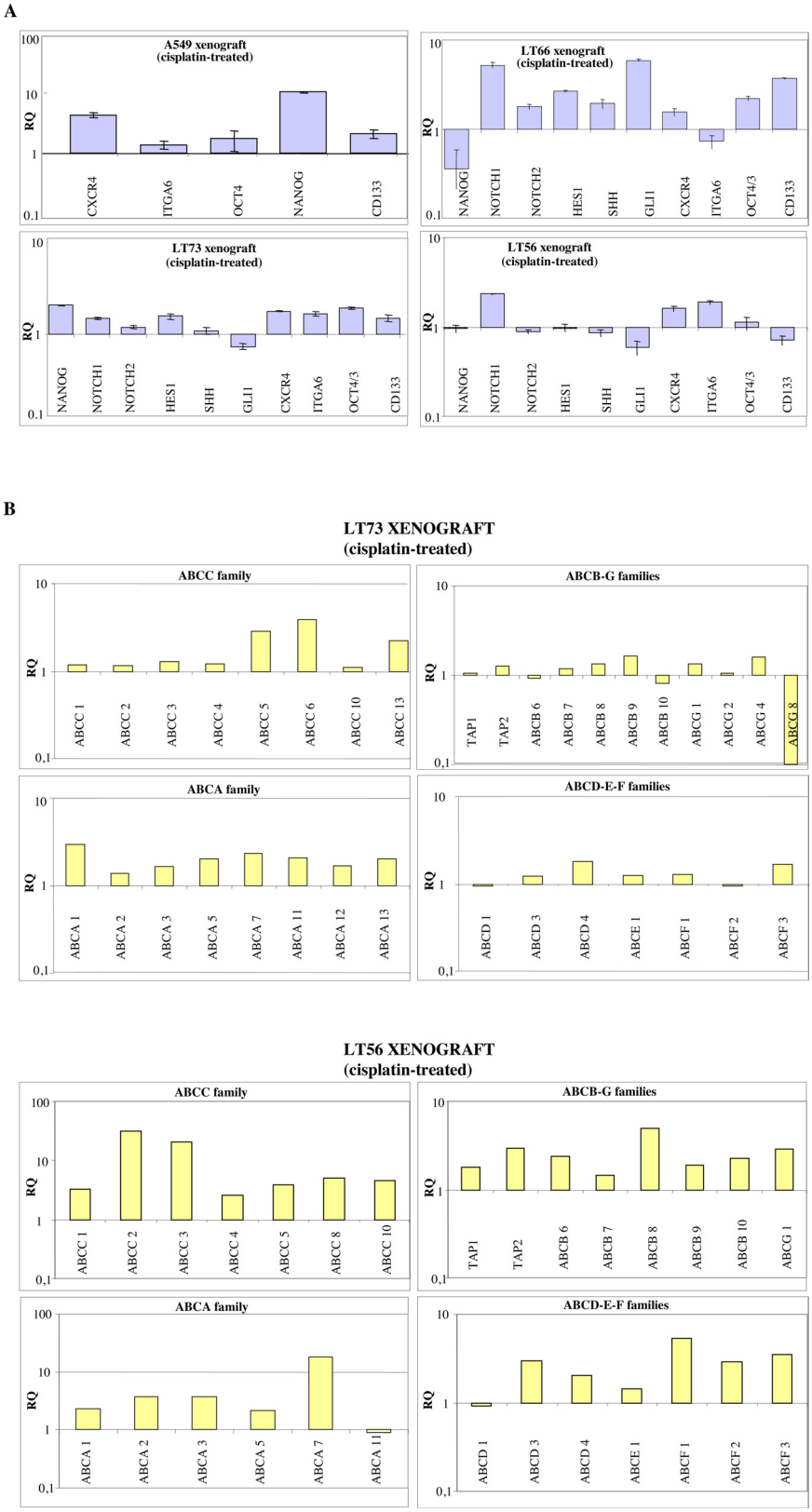

Fig. S4. (*A*) Real-time PCR analysis of stemness genes expression in cisplatin-treated A549, LT66, LT73, and LT56 xenografts. Control untreated xenografts were used as calibrator for the relative quantification of gene expression. (*B*) Real-time PCR analysis of ABC transporters in cisplatin-treated LT73 and LT56 xenografts using TaqMan Micro Fluidic cards. Control untreated xenografts were used as calibrator for the relative quantification of gene expression.

Table S1. Clinicopathological characteristics and CD133/ESA expression in NSCLC patients

ID	Histology/grading	Stage	FACS analysis CD133, %	FACS analysis ESA, %	FACS analysis CD133/ESA, %	IHC analysis CD133
LT22	ADC G3	IB	23	80	20	Positive
LT23	LC G3	IIB	31	57	15	Positive
LT24	SCC G2	IIB	1.2	12	1	Positive
LT25	SCC G3	IIIA	0	10	0	Negative
LT26	ADC G3	IIB	0.3	10	0	Negative
LT27	ADC G2	IA	0	80	0	Negative
LT28	ADC G3	IIB	2	5	/	Negative
LT29	ADC G3	IB	0	70	0	Negative
LT30	ADC G2	IB	33	89	29	Positive
LT32	SCC G2	IB	1	1.5	0.3	Negative
LT33	SCC G2	IA	/	/	/	Negative
LT34	SCC G2	IB	8	26	7	Positive
LT35	SCC G3	IA	0	42	0	Positive
LT36	ADC G3	IA	3	80	3	Negative
LT37	LC G3	IB	/	/	/	Positive
LT38	ADC G2	IA	2	13	0.7	Positive
LT39	ADC G3	IA	0.7	33	0.7	Positive
LT40	ADC G2	IIIB	0.9	70	/	Positive
LT42	ADC G3	IB	0	8	0	Negative
LT43	ADC G3	IV	2	12	2	Positive
LT44	SCC G3	IA	2	13	/	Negative
LT45	ADC G3	IIIA	36	56	35	Positive
LT46	ADC G3	IIIA	0.5	88	0.5	/
LT47	ADC G3	IIIA	2	50	2	Negative
LT48	ADC G3	IB	0.3	63	0.3	Negative
LT49	ADC G3	IIIA	3	48	3	Positive
LT50	ADC G3	IIA	2.5	35	2.5	Positive
LT51	ADC G2	IB	0	90	0	Negative
LT52	SCC G3	IA	6	43	5	Positive
LT53	SCC G3	IA	0.7	50	0.7	Negative
LT54	ADC G3	IIIB	1	78	0.7	Positive
LT55	ADC G1	IB	1.5	75	1.5	Negative
LT56	LC G3	IB	15	87	15	Positive
LT57	ADC G2	IIIA	2	57	0.7	Positive
LT58	ADC G3	IA	1	62	1	Positive
LT59	LC G3	IIIA	2	13	2	Positive
LT60	ADC G3	IA	/	/	/	Positive
LT62	SCC G3	IV	1.2	14	0.5	/
LT63	ADC G2	IA	/	/	/	Positive
LT64	ADC G3	IA	5	50	5	Negative
LT65	ADCG3	IIIB	1	46	0.5	Negative
LT66	ADC G3	IIIA	0.02	72	0.02	Negative
LT67	ADC G3	IIIA	0	70	0	Negative
LT68	SCC G2	IB	3	5	0.6	Positive
LT69	ADC G3	IIIA	1.5	84	/	Negative
LT70	SCC G3	IA	0	3	0	Negative
LT71	SCC G2	IA	1.3	6	1	Negative
LT72	ADC G2	IIIA	1	60	1	Positive
LT73	ADC G2	IB	27	93	27	Positive
LT74	ADC G2	IA	2	12	2	Positive
LT75	ADC G2	IA	12	40	12	Positive
LT76	ADC G2	IA	7.5	45	6	Positive
LT77	SCC G3	IIB	2	40	2	Negative
LT78	ADC G2	IA	0.2	10	0.2	Positive
LT79	SCC G3	IIA	3	55	3	Negative
LT80	SCC G3	IB	30	58	30	Positive
LT81	ADC G2	IV	3	20	3	Positive
LT82	ADC G2	IA	5	70	5	Positive
LT83	SCC G3	IB	1	26	1	Negative
LT84	SCC G3	IB	1.8	30	1.6	Negative

ADC, adenocarcinoma; SCC, squamous cell carcinoma; LC, large cell carcinoma. Slash (/) indicates material not available.

Table S2. Correlation of CD133 expression (by IHC) and tumor histology, stage, and grading in NSCLC patients

	IHC score						
	Overall		Negative		Positive		
	n	%	*n*	%	*n*	%	*P*
Histology							0.0441
ADC	37	63.8	15	57.7	22	68.8	
SCC	17	29.3	11	42.3	6	18.8	
LC	4	6.9	—	—	4	12.5	
Tumor stage							0.7091
Ia	20	34.5	8	30.8	12	37.5	
Ib	16	27.6	8	30.8	8	25.0	
II	7	12.1	4	15.4	3	9.4	
IIIa	10	17.2	5	19.2	5	15.6	
IIIb–IV	5	8.6	1	3.8	4	12.5	
Grading							0.0356
G1–G2	22	37.9	6	23.1	16	50.0	
G3	36	62.1	20	76.9	16	50.0	

Table S3. In vivo tumorigenicity of $CD133^+$ and $CD133^-$ cells

Case	Cell dose	Stage	Histology	CD133, %	Source (separation technique)	Tumor incidence		
						$CD133^+$	$CD133^-$	Unsorted
LT28	1×10^3	IIb	ADC	0.2	Xenograft (FC)	2/2	0/4	0/4
LT45	1×10^4	IIIa	ADC	35	Primary tumor (MB)	2/3	0/2	0/2
LT56	1×10^3	Ib	LC	18	Xenograft (MB)	3/4	0/4	2/4
LT66	1×10^3	IIIa	ADC	0.02	Xenograft (FC)	4/4	1/4	2/4
LT73		Ib	ADC	30	Xenograft (FC)			
Primary	1×10^3					3/4	3/4	3/4
Secondary	1×10^3					4/4	4/4	/
	1×10^2					4/4	2/4	/
Tertiary	1×10^3					2/2	1/2	/
	1×10^2					4/4	1/4	/
A549/s	1×10^3			0.8	Cell line (FC)	3/4	2/4	4/4
	1×10^2					5/6	1/6	4/6
A549 WT	1×10^3			0.1	Cell line	/	/	2/4
	1×10^2					/	/	0/6

FC, flow cytometry separation; MB, magnetic bead separation. $CD133^+$ and $CD133^-$ cells were sorted by FACS or magnetic beads from xenograft, primary tumors, and A549 spheres. Cells were injected s.c. into the flanks of immunocompromised mice at scalar doses. For tumor incidence, / indicates not done.

Table S4. Effect of chemotherapy [cisplatin (cisPt)] on tumor cell subpopulations

			Fraction of cells expressing, %				
Xenograft	cisPt*	Max TVI, %	CD133	CXCR4	ABCG2	CD133/ABCG2	CD133/CXCR4
A549	Control		0.3	5	36	0.3	ND
	cisPt	35	2.15	26	38	1.5	ND
	Relapse		0.7	30	ND	ND	ND
LT66	Control		0.02	1.3	33	0	0
	cisPt	70	0.7	7.8	48	0.7	0.7
	Relapse		0.15	1.3	50	0.15	0.15
LT28	Control		0.2	9.5	5.4		
	cisPt	83	0.2	19.8	24	ND	ND
	Relapse		0.15	19.5	20		
LT45	Control		50	4.5	19	14.5	ND
	cisPt	64	40	9.5	56	25	ND
LT73	Control		5.3	8.5	13.5	5	5.2
	cisPt	55	5.7	8	22	5.5	5
	Relapse		5	9	18	4.5	4.5
LT56	Control		18	7	46	6.5	3.5
	cisPt	43	17	10	68	15	7.5
	Relapse		15	7	45	7.5	3.5

ND, not determined.

*Regimen cisPt: i.v. 5 mg/kg q7d × 3 weeks.

Table S5. CD133 expression as a marker of chemotherapeutic response in advanced-stage NSCLC patients treated with carboplatin/gemcitabine: Patient characteristics

Clinicopathologic features	No. of cases (%); $n = 42$	No. of CD133$^+$ cases (IHC); $n = 10$	P
Sex			0.6966
Male	29 (69)	6	
Female	13 (31)	4	
Age			0.3049
≤61 y	19 (45)	3	
>61 y	23 (55)	7	
Smoking behavior			0.4164
Never/nonsmokers	9 (21.4)	1	
Smokers	33 (78.6)	9	
Packs per year, median (range)	18.2 (9.1–45.6)		
Stage at diagnosis			0.4164
<IV	9 (21.4)	1	
IV	33 (78.6)	9	
Histology			0.1189
Adenocarcinoma	25 (59.5)	8	
Squamous cell carcinoma	14 (33.3)	1	